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(71) Applicant: ST. JUDE MEDICAL, INC. [US/US]; One Lillehei Plaza, St. Paul, MN 55117 (US).

(72) Inventors: MIRSCH, M., William, II; 912 Transit Avenue, Roseville, MN 55113 (US). ODLAND, Thomas, L.; 6367 Deerwood Lane, Lino Lakes, MN 55014 (US). SCHANKERELI, Kemal; 7979 Neal Avenue North, Stillwater, MN 55082 (US).

(74) Agents: FINUCANE, Hallie, A.; St. Jude Medical, Inc., One Lillehei Plaza, St. Paul, MN 55117 (US) et al.

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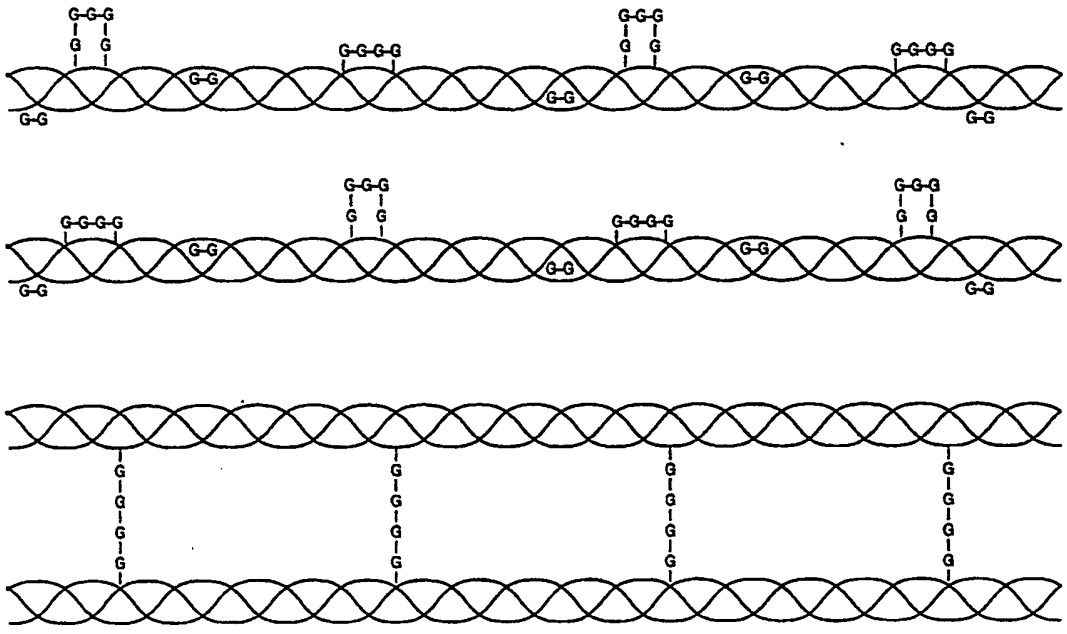

(57) Abstract

The invention concerns treating a biological tissue with a low concentration of cross-linking reagent.

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ENHANCED CROSSLINKING OF NATURAL TISSUES

TECHNICAL FIELD OF THE INVENTION

The invention concerns the use of a crosslinking agent, such as glutaraldehyde, to process a biological tissue, such as a heart valve.

BACKGROUND OF THE INVENTION

The preparation of bioprosthetic tissue prior to implantation typically includes treatment to stabilize it against subsequent *in vivo* enzymatic degradation. Typically this treatment includes crosslinking molecules, particularly collagen, on and/or in the tissue. Various aldehydes have been used for this purpose, including glyoxal, formaldehyde, and glutaraldehyde. Glutaraldehyde, however, is usually the agent of choice, in part because it may be used at physiologic pH under aqueous conditions. In addition to crosslinking the tissue, glutaraldehyde is a good sterilizing agent, and provides for reducing the antigenicity of the tissue after implantation.

Furthermore, the use of glutaraldehyde has shown to be beneficial in producing tissues of greater thermal stability, greater flexibility (in comparison to conventional crosslinking techniques), and increased durability.

However, glutaraldehyde is also cytotoxic -- even low concentrations of glutaraldehyde require rinsing the tissue to remove residual glutaraldehyde. At the concentrations typically used to crosslink biological tissues -- about 0.6% v/v, even though as little as 0.2% has been used successfully -- glutaraldehyde's toxicity is even more of a problem.

SUMMARY OF THE INVENTION

The present invention provides an improved method for crosslinking or fixing collagenous biological tissue, such as a porcine heart valve, using a low concentration of crosslinking agent. The residual levels of crosslinking agent are reduced, while maintaining or enhancing durability and flexibility of the treated tissue.

Since the process utilizes an aqueous delivery system, it avoids the use of organic solvents such as acetone, which are not only potentially toxic, but also dehydrate, denature, or destroy collagen-containing substrates (i.e., veins, arteries, heart valves, etc.). Other attributes of low-concentration glutaraldehyde fixation include: 1) a relatively higher degree of *intramolecular* crosslinks provides softer tissue. For heart valves, this means improved hemodynamics; for vascular grafts, this means greater kink resistance. 2) A relatively higher degree of short chain *intramolecular* crosslinks (along the collagen strand), which may also provide the tissue with protection from sources known to cause chain scission reactions in the collagen protein matrix. This is in contrast to long chain intermolecular crosslinks (between collagen strands) which are prevalent in high concentration glutaraldehyde crosslinking. 3) Resistance to calcification. Calcification of bioprosthetic products, such as bioprosthetic heart valves, is a very complex process with a large number of contributing factors. One of these is that calcification tends to develop in the presence of residual free glutaraldehyde. Fixation in low concentrations of glutaraldehyde typically diminishes the amount of residual glutaraldehyde in the tissue. Furthermore, while calcification also tends to

manifest itself in the vicinity of tissue fractures, softer tissue resulting from low-concentration crosslinking is less likely to develop fractures or cracks over time.

While the present invention is suitable for a number of vascular prostheses, it is of particular value in the field of small diameter vessel replacement. It is useful in coronary access bypass procedures, particularly when the patient has had previous coronary replacement surgery and adequate saphenous veins or internal mammary arteries no longer exist due to previous excision. Since the prosthesis made in accordance with this invention may have patency equivalent to or better than that of a saphenous graft, the use of such a prosthesis would alleviate the need for saphenous vein excision. It is also applicable to systemic microvascular vessel replacement, such as in the hand or foot.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 compares tensile strength values of bovine carotid arteries crosslinked in 0.01% glutaraldehyde vs. 0.05% glutaraldehyde.

Figure 2 compares suture retention values of bovine carotid arteries crosslinked in 0.01% glutaraldehyde vs. 0.05% glutaraldehyde.

Figure 3 shows the effect of different fixation conditions on tensile and suture retention strengths of bovine median arteries.

Figure 4 shows the effect of different fixation conditions on bursting strength of bovine median arteries.

Figures 5a and 5b illustrate the difference between low concentration glutaraldehyde intramolecular

crosslinks and high concentration glutaraldehyde intermolecular crosslinks, respectively.

Figure 6 compares the leaflet shrink temperature of leaflets crosslinked in 0.5% glutaraldehyde vs 0.05% glutaraldehyde.

Figure 7 shows the effects of e-beam radiation on pressure drop.

Figure 8 shows the effects of e-beam on effective orifice area.

Figure 9 shows shrink temperature versus fixation time for aortic root tissue.

DETAILED DESCRIPTION OF THE INVENTION

A method in accordance with the invention includes treating or processing biological tissue by exposing the biological tissue to less than about 0.1% by volume crosslinking solution, preferably between about 0.01% to about 0.099% by volume fixing solution. In a preferred embodiment of the invention, the fixing solution is a buffered solution containing glutaraldehyde.

The term "biological tissue" as used herein refers to a collagen-containing material which may be derived from different animal species, typically mammalian. The biological tissue is typically a soft tissue suitable for implantation, such as bioprosthetic tissue or the like, but the invention should not be limited thereby. Specific examples include, but are not limited to, heart valves, particularly porcine heart valves; aortic roots, walls, and/or leaflets; pericardium, preferably bovine pericardium or the like, and products derived from pericardium, such as a pericardial patch; connective tissue derived materials such as *dura mater*; homograft tissues, such as aortic homografts and saphenous bypass grafts; tendons,

ligaments, skin patches; blood vessels, particularly bovine arteries and veins, and human umbilical tissue, such as veins; bone; and the like. Any other biologically-derived materials which are known, or become known, as being suitable for processing in accordance with the invention are within the contemplation of the invention.

In accordance with the invention, the biological tissue, explanted from its source, may be processed in any suitable manner prior to exposure to a crosslinking agent. Typically, the biological tissue is carefully cleaned and then treated with a filtered proteolytic enzyme concentrate to digest and thereby substantially eliminate antigenic tissue from the biological tissue. When natural tissue is utilized, the cleaning step typically involves stripping the adventitia and undesirable fat and muscle tissue from the tissue.

Exposing the biological tissue to a crosslinking reagent, as used herein, refers to any method of contacting the biological tissue with the crosslinking agent. In a preferred embodiment of the invention, exposing the tissue to a crosslinking agent refers to immersing the tissue in the crosslinking agent.

When tissue is immersed in a solution of a given concentration of a crosslinking agent such as an aldehyde, e.g., glutaraldehyde, the concentration of glutaraldehyde within the interstices of the tissue equilibrates with the surrounding solution so that the tissue experiences true low-concentration crosslinking. However, when glutaraldehyde is delivered via a nebulizer, monomeric glutaraldehyde is deposited directly to the surface of the substrate. Regardless of

the initial concentration of the solution prior to vaporization, pure glutaraldehyde is in contact with the tissue. The concentration in the tissue is therefore very difficult, if not impossible, to control.

The glutaraldehyde that is typically used in accordance with the invention is a biological grade 50% solution commercially available, from, for example, Electron Microscopy Sciences (Fort Washington, PA). Such commercially available glutaraldehyde may also be available in a variety of other grades, purities, and/or concentrations. A biological grade of glutaraldehyde typically does not require additional purification, but it may be desirable to pass the final crosslinking solution through a 0.2μ pore size hydrophilic membrane filter to remove any biological contaminants.

In accordance with the invention, a biological tissue may be exposed to a fixing solution comprising glutaraldehyde in a suitable buffer. Suitable buffers for use in the practice of the invention are those buffers which have a buffering capacity sufficient to maintain a physiologically acceptable pH, do not cause substantial deleterious harm to the biological tissue, and/or do not interfere with the treatment process. Exemplary buffers include, but are not limited to phosphate-buffered saline (PBS), and organic buffers, such as N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) or morpholine propanesulphonic acid (MOPS); and buffers which include borate, bicarbonate, carbonate, cacodylate, or citrate. The preferred fixing solution is less than 0.1% v/v glutaraldehyde in a citrate buffer.

In a typical protocol according to the invention, the biological tissue may be exposed to the fixing solution for a time and at a temperature

sufficient to induce crosslinking of the collagen in and on the biological tissue. For example, the biological tissue may be exposed to a buffered glutaraldehyde solution from about 4°C to about 37°C, preferably at about 20°C; at a pH from about 6 to about 8, preferably about 6.3 to about 6.5; and for a period up to about 10 days, preferably from about 2 to about 5 days.

In accordance with the invention, one skilled in the art will recognize that certain parameters in the treatment protocol may be varied according to achieve a particular purpose. These parameters include, but are not limited to glutaraldehyde concentration, solution composition, pH and ionic strength, time and temperature of biological tissue exposure to glutaraldehyde, and the ratio of tissue to volume of solution, and the biological tissue configuration during the initial fixation. The invention is not to be limited thereby.

Typically, the crosslinked biological tissue is then rinsed, using, for example, any suitable rinsing or laving material. In a preferred embodiment, the rinsing agent is sterile, physiological saline.

The tissue may be rinsed with many volumes of sterile, physiological saline over a period of approximately 24 hours, or until the concentration of residual processing chemicals in the tissue are below levels which are considered to be toxic (approximately 1 ppm).

One skilled in the art will also recognize that the invention may include additional processing. For example, the crosslinked biological tissue may be exposed to one or more anticalcification agents, one or more bioburden reduction agents, at least one rinsing solution, and one or more sterilizing agents or protocols. Furthermore, as shown in more detail below,

the biological tissue, crosslinked in accordance with the invention, may be stored for up to about one year or more prior to final sterilization. Exemplary additional processing is described in more detail below.

BIOBURDEN REDUCTION

An embodiment of the invention may include exposing the crosslinked biological tissue to one or more bioburden reduction agents, typically for up to about 10 hours, preferably for about 2 to about 4 hours. For example, a porcine heart valve treated with glutaraldehyde as noted above may then be exposed to a buffered solution containing about 1-5% glutaraldehyde, about 1-6% formaldehyde, and about 15-25% ethanol. Typical buffers include PBS, HEPES, phosphate, and citrate buffers.

INTERMEDIATE STORAGE

An embodiment of the invention may include storing the crosslinked biological tissue for up to a year or more prior to final sterilization. For example, a porcine heart valve crosslinked with glutaraldehyde in accordance with the invention may be temporarily stored at room temperature in a 0.5% glutaraldehyde solution buffered with PBS, citrate or the like.

ANTICALCIFICATION TREATMENT

An embodiment of the invention may include exposing the crosslinked tissue to one or more reagents designed to reduce or inhibit calcification of the biological tissue after implantation. A number of anti-calcification reagents are known in the art. For example, the crosslinked biological tissue may be exposed to an alcohol and/or an aluminum salt in order to reduce or inhibit calcification. In an exemplary process, the crosslinked biological tissue may be immersed in a solution containing greater than about 50%

of a lower aliphatic alcohol for a period sufficient to render the biological tissue resistant to calcification, typically up to about 96 hours.

The alcohol is preferably a lower aliphatic alcohol (C1 to C3), such as methanol, ethanol, propanol or isopropanol. In a preferred embodiment, the alcohol is ethanol.

The length of time for exposure to the alcohol treatment can be varied by those of skill in the art. For embodiments of the invention wherein the biological tissue is immersed, or soaked, in a liquid treatment solution of the alcohol, an illustrative exposure time is preferably between about 24 to 96 hours.

In another embodiment of the invention, the anticalcification agent may include a multivalent metallic cation, such as a salt of aluminum or iron. For example, the crosslinked biological tissue may be immersed in a solution containing from about 0.1M to about 0.001M $AlCl_3$ for a period sufficient to render the biological tissue resistant to calcification.

In accordance with the invention, the crosslinked biological tissue may be stored in an alcohol-glutaraldehyde solution, preferably in an amount sufficient to maintain calcification inhibition and/or sterility. For example, the biological tissue may be stored in a buffered alcohol solution containing glutaraldehyde, typically greater than about 60%, and preferably between about 60% and about 80%, alcohol and less than about 0.5%, preferably between about 0.2% to 0.5%, glutaraldehyde.

The biological tissue, calcification-inhibited and/or crosslinked, may then be placed or packaged in a container. In accordance with a preferred embodiment of the invention, the biological tissue is packaged and

sealed, in physiological saline, in its final container prior to terminal sterilization. Packaging preferably means placing in a container suitable for sterilization, storage, and/or shipping.

The container may be constructed of glass or polymeric plastic, such as polypropylene, polyethylene, and/or epoxies. It is intended that the invention should not be limited by the type of container and seal being employed; other materials may be used, as well as mixtures, blends, and/or copolymers.

The crosslinked, packaged biological tissue may then be sterilized, as noted below, or it may be stored for up to about a year or more prior to sterilization. In accordance with the invention, storage includes long term storage, e.g., six months, twelve months, or for up to about five years or more.

STERILIZATION

A method in accordance with the invention may also include sterilizing the tissue. The term "sterilizing" as used herein refers to exposing the biological tissue to a sterilizing beam of accelerated electrons, i.e., an electron beam (e-beam). The particle beam which comprises the e-beam preferably includes directional bombardment, i.e., bombardment from one direction only, and includes single-side or multiple-side irradiation. The biological tissue, crosslinked in accordance with the invention, may be sterilized, preferably after the biological tissue has been packaged. Suitable sterilizing protocols include, but are not limited to x-ray or gamma radiation, e-beam radiation, and the like. The preferred method of sterilizing the crosslinked tissue is by exposing the biological tissue, packaged in saline, to accelerated electrons. For example, the biological tissue may be

subjected to the electron beam until a dose of approximately 25 kilogray (kGy) is achieved, or approximately 1-10 minutes, depending on the dimensions of the material.

A major advantage of e-beam processing over conventional gamma radiation is the processing speed or the high rate at which the energy can be applied in a controlled manner, which usually translates to lower sterilization costs.

In accordance with the present invention, biological tissues are treated by exposing the tissue to e-beam radiation sufficient to effect sterilization. Additionally, the present invention provides a biological tissue sterilized by e-beam radiation, with the resulting biological tissue exhibiting enhanced performance characteristics. The methods and tissues according to the present invention have the added advantage of reduced risk of infectivity, and eliminates the need for aseptic handling protocols. Further, the methods and tissues of the present invention, which use fewer reagents and/or require less processing, provide for lower costs in labor, reagents, time and personnel.

E-beam radiation sterilization is effective in obviating the need for toxic sterilizing chemicals. Moreover, the amount of radiation required for e-beam sterilization does not significantly degrade the biological tissue, thus providing a more durable transplantable tissue.

The dose rate for gamma radiation is approximately 110 grays per minute and the dose rate of e-beam is approximately 7800 grays per minute. Consequently, exposure times are dramatically greater for gamma radiation, which requires low doses over an extended period to effect sterilization. In

contradistinction to gamma radiation, the high dose rates involved in e-beam irradiation promote diffusion of oxygen into biological tissue at a rate insufficient to participate in free radical formation reactions, such as those which might contribute to tissue and polymer degradation. This is particularly advantageous in those embodiments which include placing the biological tissue in a container prior to irradiation, since polymer degradation in both the tissue and the container may be minimized.

Furthermore, the high dose rate of e-beams relative to gamma rays permits a higher processing rate of sterilization, commonly an order of magnitude higher.

In relative terms, gamma radiation penetrates approximately ten times further into materials than 10 MeV electrons in the same material.

Gamma rays induce excitation of electrons *within* the atoms of the materials to be sterilized. Electron beams, on the other hand, provide high-energy electrons to the exterior of the material, which penetrate the material, and in turn put subsequent electrons in motion.

Because of the relatively high dose rate of the e-beams, oxygen is not capable of diffusing into the material at a rate required to participate in oxidative reactions that may lead to degradation of the material.

Furthermore, prevention of degradation in both the package and the tissue permit terminal sterilization, i.e., sterilization of the tissue in its final, sealed package. Thus, the present invention avoids the need for costly aseptic handling techniques, and provides sterility assurance as long as the package is intact, i.e., until the tissue is ready for use.

As high energy electrons penetrate the surface they collide with atomic electrons of the material. These electrons, in turn, recoil and collide to set more electrons in motion so that from a relatively few electrons penetrating the surface, there results a multiplicity of electrons depositing energy in the material, primarily by the production of ions and free radicals. This process, called buildup, results in higher doses being delivered to depths below the surface where the primary beam and its recoil electrons can no longer produce ionization.

In accordance with the invention, the amount of e-beam radiation is an amount sufficient to sterilize the biological tissue, and in some embodiments, an amount sufficient to sterilize the biological tissue packaged in its final container. One skilled in the art will recognize and be able to determine a sterilizing dose and time suitable for a particular tissue and based on the characteristics of the accelerator being used.

Typically, the biological tissue is subjected to a one-sided exposure to the electron beam until a sterilizing dose of radiation is absorbed. Absorbed dose of radiation is expressed in terms of kilograys (Kgy). One kilogray is equal to one thousand joules of energy deposited per kilogram of material. For example, the biological tissue may be irradiated until a dose of approximately 25 Kgy or more is achieved.

Effective sterilization may be easily determined using conventional microbiological techniques, such as for example, the inclusion of suitable biological indicators in the radiation batch or contacting the tissue with a culture medium and incubating the medium to determine sterility of the tissue. Dose may also be determined with the use of

radiochromic dye films. Such films are calibrated, usually in a gamma field, by reference to a national standard.

Degradation of the biological tissue by irradiation may also be determined using well known and conventional tests and criteria, e.g. reduction in shrink temperature, T_s; susceptibility to enzyme attack, e.g. collagenase; extractability of degradation products, e.g. collagen fragments; and decrease in physical properties such as tensile strength.

In a typical protocol according to the invention, the biological tissue may be exposed to the fixing solution for a time and at a temperature sufficient to induce crosslinking of the collagen in and on the biological tissue. For example, the biological tissue may be exposed to a buffered glutaraldehyde solution from about 4°C to about 37°C, preferably at about 20°C; at a pH from about 6 to about 8, preferably 6.3 to 6.5; and for a period up to about 10 days, preferably from about 2 to about 5 days.

The aforementioned steps, in combination, produce a prosthesis having greater strength and pliability, reduced antigenicity, and greater ease of use than prostheses produced using other processes.

LONG TERM STORAGE

In another embodiment of the invention, the biological tissue, crosslinked in accordance with the invention, may be packaged in a solution of approximately 0.5% glutaraldehyde. Using aseptic techniques, the biological tissue may be thoroughly rinsed with sterile, physiological saline (e.g., 0.9% sodium chloride). The purpose of the saline rinse is to reduce the concentration of residual glutaraldehyde present on the tissue surface and in the interstitial

spaces of the tissue, thus minimizing the chance of a toxic response in the patient. The biological tissue may be placed into a plastic or polymeric container, filled with sterile saline, and capped or sealed. The capping of the product should be a permanent seal that should not be opened until the time of the implant.

The present invention provides an improved biological tissue in view of its strength, pliability, and reduced antigenicity. These characteristics are particularly desirable in view of the stressful procedure required to implant the biological tissue and the possibility of rejection by the body. With respect to the implantation procedure for some biological tissues, the biological tissue must have resistance to kinking, good suture puncturability, and the ability to readily seal suture holes. Suture retention, i.e., the ability to resist tensile force, must be high. Additionally, the graft must have high resistance to bursting to withstand possible high systolic pressures and to guard against aneurysm formation. Grafts that are insufficiently strong and/or are too antigenic may be potentially fatal to the patient.

The prostheses produced according to the present invention may be packaged as a kit, preferably sterile wet-packaged in a 0.9% NaCl solution. The selection of such additional elements are well within the ordinary skill in the art.

EXAMPLES

Example 1. Fresh tissue (e.g., blood vessels, hearts, heart valves, or pericardium) are procured from a local processing facility (bovine, porcine, ovine, etc.) and received in physiological saline (0.9% sodium chloride) on ice. The tissue is either dissected immediately or placed in fresh sterile saline and refrigerated

overnight. Extraneous tissue such as adipose, skeletal muscle, myocardium, bone, trachea, etc., is carefully removed from the tissue of interest. The tissue is then again washed and immersed in fresh sterile saline.

Although this technology works to varying degrees at a range of glutaraldehyde concentrations, approximately 0.03% v/v provides radioprotective properties and the crosslinking time fits reasonably well within a manufacturing schedule. For 10.0 liters of 50 mM citrate buffered 0.03% (v/v) glutaraldehyde:

Step 1)

A 50 mM citrate buffer solution is prepared per the following formula (10 liters):

To 9.0 liters of sterile, de-ionized water, add:

140.0 grams of Sodium Citrate

5.0 grams of Citric Acid Monobasic

38.6 grams of Sodium Chloride

Bring the volume of the solution up to 10.0 liters with sterile, de-ionized water

Step 2)

To 9.0 liters of the 50mM citrate buffer solution prepared in Step 1, add 6.0 milliliters of 50% Biological Grade Glutaraldehyde

Bring the solution volume up to 10.0 liters using the 50mM citrate buffer solution prepared in Step 1.

Step 3)

Adjust the pH of the solution to 6.40 $\pm$ 0.05 using hydrochloric acid or sodium hydroxide.

The tissue is then immersed in the glutaraldehyde solution, at room temperature (20-25°C) for the crosslinking reaction. As fixation time progresses, the number of crosslinks increases, as shown in the form of a shrink temperature curve (See Figure 9). The concentration of glutaraldehyde in solution decreases as it is consumed by the tissue in the form of poly-glutaraldehyde crosslinks. Therefore, it may be desirable to replenish the fixation solution at intervals throughout the crosslinking reaction. Because a majority of the crosslinks are formed early, it is recommended to change the solution approximately eight hours following the onset of the reaction, then daily thereafter.

The exposure of tissue to the glutaraldehyde solution proceeds for a period of time ranging from about 24 to about 120 hours, depending on the concentration of glutaraldehyde in the solution. In general, a high glutaraldehyde concentration corresponds to a short fixation time; a low glutaraldehyde concentration corresponds to a long fixation time. For a 0.03% solution, an exposure time of approximately 72 hours is sufficient to maximize the crosslink density within the interstices of the tissue. This corresponds to a shrink temperature of approximately 80-89°C, depending on the type of tissue used.

When the crosslinking reaction has ended, the tissue is submersed in a sterilant solution containing 2% (v/v) glutaraldehyde, 3% (v/v) formaldehyde, and 20% (v/v) ethyl alcohol. This multi-component sterilant reduces any residual bioburden on the tissue prior to rinsing and packaging.

The tissue is then thoroughly rinsed with sufficient sterile saline to minimize the presence of

the processing chemicals. This typically requires applying four or five 10 liter aliquots over a 24-hour period. The exposure time should be watched carefully, since diffusion of residuals from the tissue is a time-dependent phenomenon. After the final rinse, the tissue is placed in a sterile container (valve jar, vascular graft vial, etc.) and filled with sterile saline. The package is then permanently sealed. Note: all manipulations of the tissue subsequent to the bioburden reduction process with the multi-component sterilant should be performed as aseptically as possible to minimize the extent of contamination prior to e-beam sterilization.

Example 2. Two identical experiments were performed to evaluate the effects of different concentrations of glutaraldehyde on the physical properties of bovine carotid artery vascular protheses. Arteries were received in cold, sterile saline (0.9% sodium chloride). Extraneous tissue such as adipose, bone, cartilage, connective tissue, etc., was stripped from the vascular tissue. The arteries were enzymatically digested with a citrate-buffered ficin solution to remove a specified portion of smooth muscle tissue. The arteries were thoroughly rinsed and placed into one of two tanks containing glutaraldehyde to crosslink the collagen component of the tissue. One tank contained 50mM citrate-buffered 0.01% glutaraldehyde, the other tank contained 50mM citrate-buffered 0.05% glutaraldehyde. The arteries in the 0.01% solution were allowed to crosslink for approximately five days and the arteries in the 0.05% solution were crosslinked for approximately two days. Upon completion of the crosslinking reaction,

all arteries were placed in a 50mM citrate-buffered 2% glutaraldehyde solution as a sterilization step.

Tissue samples were then submitted for evaluation of Radial Tensile Strength and Suture Retention Strength. The Radial Tensile Strength test involves cutting a piece of crosslinked carotid artery, making a longitudinal incision, and pulling the tissue in a radial orientation until failure on an apparatus such as an Instron. The Suture Retention Strength test involves inserting a loop of surgical suture, such as a 5-0 PTFE-impregnated polyester suture, through a tissue sample a specified distance from a cross-sectionally cut edge. This distance, the bite size, may be, for example, about 3 mm. The suture is pulled until the tissue fails on an apparatus such as an Instron. The results of the Tensile Strength and Suture Retention Strength testing is displayed on Figure 1 and 2, respectively.

The results of the Tensile Strength testing show there are no significant differences in the strength of the tissue that can be attributed to variation in fixation solution concentration. The seemingly wide error bars are due to the inherent diversities found in biological tissues.

There appears to be a trend in the Suture Retention Strength, specifically, strength for both concentrations is lower in Batch 2 and Batch 1. This is most likely a result of a difference in the enzymatic digestion processes. Each ficin solution varies slightly in enzymatic activity. In other words, it is possible that the digestion solution prepared for Batch 2 had a slightly higher enzyme activity than that for Batch 1. Nevertheless, the differences in strengths *within* the batch are negligible.

Example 3. An experiment was performed to evaluate the effects of different concentrations of glutaraldehyde on some of the physical properties of bovine median artery vascular protheses. Processing of tissue was the same as that described in Example 2 until the crosslinking step. The digested arteries were placed in one of three tanks for crosslinking. Tank 1 contained 50mM citrate-buffered 0.01% glutaraldehyde, tank 2 contained 50mM citrate-buffered 0.075% glutaraldehyde, and tank 3 contained 50mM citrate-buffered 2.0% glutaraldehyde. The arteries were allowed to remain in each of the fixation tanks until the crosslinking reaction was completed, or about four days. Upon completion of the crosslinking reaction, half the arteries from tanks 1 and 2 were placed in a 50mM citrate-buffered 2% glutaraldehyde solution as a sterilization step. The other half remained in the original solution. After 24 hours, all arteries were placed in individual glass vials containing 40% ethyl alcohol, and capped. Samples from each group were then subjected to the following test: Radial Tensile Strength, Suture Retention Strength, and Bursting Strength.

Figure 3 shows that the radial tensile strength and suture retention strength are very similar for tissue processed at each of the conditions in this experiment. The extremes (0.01% and 2.0%) are of particular interest because this data represents the lower end of the range. This data contradicts the very common notion in the industry that maintains that high concentration fixation leads to high thermal stability, which leads to high strength. These results, as well as the shrink temperature data in Figure 6 show that this is not the case. The fact that the 0.01%/2% group has a sightly lower tensile strength than the other groups

is considered an artifact. It is expected that the subsequent treatment with 2% glutaraldehyde will, if anything, *strengthen* the tissue, not weaken it.

Figure 4 shows the results of the Bursting Strength testing, where a vascular prosthesis sample is inflated with water until it bursts. The internal pressure at the time of failure is recorded as the bursting strength. Again, looking at the extremes, results are nearly identical, even though the low-glutaraldehyde test group was fixed at a glutaraldehyde concentration 1/200th of conventional 2% crosslinking.

Example 4. Figures 5a and 5b are simplified representations of the formation of a short-chain *intra*molecular crosslinks produced by low-glutaraldehyde and long-chain *inter*molecular crosslinks produced by high-glutaraldehyde fixation, respectively. These Figures aid in showing that, in the presence of intramolecular crosslinks, the collagen strand may maintain much of its integrity, even if peptide bonds are cleaved by radiation exposure.

It is possible to analyze the crosslink density of tissue by measuring the shrink temperature, or denaturation temperature, of a given sample. Tissue crosslinked with low glutaraldehyde forms a higher density of intramolecular crosslinks, and this may be expressed in terms of a higher shrink temperature than conventionally-fixed tissue. This relationship is shown by crosslinking two groups of fifteen porcine aortic valve leaflets with 0.5% or 0.05% glutaraldehyde. Shrink temperatures were measured via Differential Scanning Calorimetry and the results are contained in Figure 6.

Example 5. Experiments have shown that glutaraldehyde-crosslinked tissue, exposed to e-beam radiation, exhibits enhanced hemodynamic performance characteristics, such as flexibility. Evidence of increased flexibility is provided by measuring pressure drop across the heart valve (the change in pressure from the inflow side of the valve to the outflow side), as shown in Figure 7. Enhanced flexibility is also shown by measuring the effective orifice area, the cross sectional area through which blood flows, as shown in Figure 8. These tests show that exposing heart valves to e-beam radiation results in softer leaflets which tend to open more readily and to a greater extent than non-irradiated valves. This provides both short-term and long-term benefits to the recipient because a larger effective orifice area results in greater cardiac output and therefore, an increase in efficiency of cardiac activity and a decreased tendency to develop cuspal fractures leading to eventual calcification and valve failure.

Eight heart valves were glutaraldehyde crosslinked as shown in Example 1 and exposed to e-beam radiation. The pressure drop across the heart valve before subjecting the heart valve to e-beam radiation was compared to the pressure drop after subjecting the heart valve to E-bean radiation. Figure 7 graphically illustrates that the pressure drop decreases when tested on a steady state in vitro flow tester. As a reference point, the pressure drop for a straight, unobstructed tube would be zero.

Figure 8 compares the effective orifice area before and after exposing the heart valve with e-beam radiation, and shows that the effective orifice area increases following e-beam radiation.

Effective Orifice Area determinations were made by placing test valves in a Pulse Duplicator system. The Pulse Duplicator is capable of calculating a number of valve-related functions by measuring pressures and flow rates at strategic locations within a simulated heart containing the test valve.

Effective Orifice Area (EOA) is defined as follows:

$EOA = Q_{rms}/(51.6\sqrt{\Delta P})$, expressed in cm^2, where

Q_{rms}= root mean square flow rate obtained during the period of positive pressure drop, in ml/second

ΔP= mean positive pressure drop, in mm Hg

The theory behind enhanced hemodynamics in irradiated tissue heart valves involves the disruption of molecular bonds which hold the collagen triple helix intact. The intramolecular crosslinks offered by this technology serve as reenforcement to the collagen backbone as its own structural frame work is weakened by the radiation. A dose of 25 kGy, in the presence of sufficient intramolecular crosslinks, weakens the protein framework to sufficiently render the tissue more flexible, yet the tissue performance improves.

Similar results have been obtained every time these two experiments were repeated. While the exact mechanism is unknown, it is theorized that a scission reaction occurs within the collagen molecule. Bonds that hold the collagen chain together appear to be broken when subjecting a tissue to e-beam radiation. However, the presence of intramolecular glutaraldehyde crosslinks appears to keep the primary structure of the

collagen molecule intact, thus maintaining the integrity of the softened tissue.

Example 6. The major criticism of radiation as a sterilization method for biological tissues is its effect on long-term durability of the product. The FDA currently requires that tissue valves demonstrate the ability to withstand 200 million cardiac cycles on an accelerated wear tester. This translates to approximately five years of real time. At some point in the future, 380 million cycles of the same testing may be required.

An experiment was performed an experiment to determine the effects of e-beam radiation on the wear-resistance of tissue valves. Four groups of valves were tested:

Group 1 Crosslinked in 0.03% glutaraldehyde; stored in 0.5% glutaraldehyde (e-beam negative control).

Group 2 Crosslinked in 0.03% glutaraldehyde; rinsed for removal of residuals; stored in 0.9% sodium chloride; e-beam sterilized, 25 kGy.

Group 3 Crosslinked in 0.03% glutaraldehyde; treated with anticalcification process; rinsed for removal of residuals; stored in 0.9% sodium chloride; e-beam sterilized, 25kGy.

Group 4 Crosslinked in 0.5% glutaraldehyde; rinsed for removal of residuals; stored in 0.9% sodium chloride; e-beam sterilized, 25 kGy (concentration negative control).

Results of this experiment are located in Table 1 below. These results clearly indicate that, compared to control valves (Groups 1 and 4), exposing the tissue valves to e-beam radiation does not have a negative effect on durability after *in vitro* testing at 389 million cardiac cycles. The group with the best wear data, in fact, was the group that had been exposed to e-beam after a treatment for anticalcification.

Table 1.

Results of 389 Million Cycles Accelerated Wear Testing:

e-beam v. No e-beam

Treatment	Number of Valves	Summary of Anomalies
Group 1	4	6 large holes (> 1mm) 2 small holes (≤ 1mm) 2 large tears in leaflets (2-6 mm) 1 small abrasion 1 valve with no observed wear
Group 2	4	2 large holes 5 small holes 1 small abrasion 1 valve with no observed wear
Group 3	6	3 small holes 4 valves with no observed wear
Group 4	3	3 holes (0.5 to 3 mm) 2 valves with no observed wear

Example 7. A vascular graft prosthesis, for example an artery, is stripped of its adventitia, fat and muscle tissue, and digested in an activated protease. This stripped and cleaned artery is treated with succinic anhydride at a controlled basic pH to produce a negative charge on the surface of the artery. The negatively charged artery may then be treated with glutaraldehyde in a concentration ranging from about 0.005 to about 5%

in a citrate buffer to fix and strengthen the artery by cross-linkage. The preferred concentration is less than about .01%. In accordance with the present invention, low concentrations of glutaraldehyde typically yield softer and more flexible products, based on qualitative comparative assessments of graft products cross-linked using low and high glutaraldehyde concentrations. Qualitatively, increased pliability of graft products crosslinked using low glutaraldehyde concentrations was demonstrated by assessing the radius of curvature of the graft; the radius is smaller in low glutaraldehyde concentration fixed product than in a high glutaraldehyde concentration fixed product.

The fixed graft may then be sterilized using glutaraldehyde at a concentration range of about 1% to about 4%, preferably about 2%.

This combination of steps produces a prosthesis having increased strength, increased durability, increased pliability, and decreased thrombogenicity.

The digestion step in the present invention comprises using an activated protease to remove antigenic material from the surface of the graft. While it is known to utilize the proteolytic enzyme ficin as part of the cleaning step in preparing a blood vessel, the present method provides more effective elimination of antigenic tissue from the natural graft prosthesis through the use of an activated protease. What remains after the digestion step is a collagen matrix.

Example 8. After harvesting, the graft is digested in a protease, preferably, an activated protease, to remove antigenic substances from the graft. In a preferred embodiment, the activated protease is ficin, more

preferably, filtered ficin that is activated by adding cysteine to the ficin concentrate. In a preferred embodiment, a buffer is used comprising citric acid and sodium citrate, although other buffers may be used.

The specific amount of ficin used in the practice of this invention depends on the activity of the ficin used, since variances in the activity level of enzymes are normal. To make up differences in the activity levels of the ficin used, the exact amount of ficin required to manufacture grafts is typically adjusted each time grafts are manufactured to achieve a constant volumetric activity; i.e., the determination of the specific quantities of ficin to be used during graft production typically requires that the ficin be evaluated for activity and that adjustments to the amount of ficin used be made in accordance to the activity noted. The concentration of ficin typically used according to the present invention amounts to the addition of approximately 65 grams of ficin to a reactor volume of about 20 liters. In a preferred embodiment, the ficin activity of the digesting solution is about 9.8 mmol NPZG/liter-minute.

During digestion, the temperature and pH should be monitored. In one embodiment, the temperature should be in the range between 30^0C and 50^0C, more preferably 40^0C $\pm$ 2^0C. The pH should be in the range between 5 and 7, more preferably 6.3 $\pm$ 0.1. The digestion time utilized is not critical, but should be sufficient to remove any antigenic material; 2-3 hours is typically sufficient. The graft should then be rinsed several times in distilled water. In a preferred embodiment, the graft is rinsed 4 times. Digestion is terminated in a stop bath, preferably a stop bath containing sodium chlorite. In a preferred embodiment,

the concentration of sodium chlorite is 0.1%. The graft is then rinsed again, preferably 4 times.

Example 9.

Using a hemostat, suspend one artery from a ring stand. Using a clean scissors, strip adventitia, fat, and muscle tissue from the artery. Place the artery in a clean beaker with clean 0.9% sodium chloride on ice. Repeat above steps until all arteries have been stripped.

Tie off side branches with a suture. Tie off as close to the main artery as possible with a triple surgeon's knot. Trim excess side branches near the knot. Remove the artery from the ring stand when all side branches have been tied.

Fill a large syringe with 0.9% sodium chloride solution and attach the proper leak test fitting to the luer tip. Using a hemostat, clamp one end of the artery. Insert the tip of the syringe into the opposing end of the artery. While securing the end of the artery to the syringe tip with one hand, gently inject saline into the lumen of the artery to detect leaks. Close any leaks with a 3-0, 4-0, or 5-0 suture. Tie off the suture with a triple surgeon's knot. Continue to suture leaks in the artery until it is leak tight under moderate pressure on the syringe. Place sutured artery in a clean beaker with clean 0.9% sodium chloride solution on ice. Repeat above steps until the desired number of arteries have been sutured.

Dissolve 420 grams of sodium citrate and 14.4 grams of citric acid in H_2O and adjust the volume to 21 liters. Measure the pH and adjust to 6.3 if necessary. The overall citrate concentration is 71.3 mM with 95.4% sodium salt and 4.6% acid form.

Fill the reactor with 17.32 liters of ficin buffer and turn the mixer on. Place the heater coil in the tank and turn the hot water on to start heating the buffer to 40°C. Insert the threaded hose barb fittings into the arteries and use cable ties to fasten them. Attach the arteries to the graft manifold using the threaded fittings. Attach the graft manifolds to each other and to the graft manifold support assembly using 3/8"x2" stainless steel pegs and place in the reactor. Start the pumps with a flow rate of 1.05 liters/minute through each manifold. Add the activated ficin concentrate solution when the reactor temperature stabilizes at 40°C.

The concentrated ficin solution is made to the equivalent of 85 grams/liter of Sigma latex powder. Place 2.8 liters of ficin buffer in a flask in the 40°C water shaker bath. Approximately 65 grams of ficin powder are required. The ficin powder is dissolved in the 40°C buffer and filtered through a 5 micron Gelman Acro 50A filter.

Measure out 2.68 liters of the filtered enzyme concentrate into a flask and place in the 40°C water bath. Weigh out 6 grams of L-cysteine and add to the enzyme concentrate for activation. After 5 minutes, add the enzyme concentrate to the reactor and begin digestion. Digestion takes place for 2½ hours. Temperature is maintained at 40°C +/- 2C° and the pH is monitored and should remain at 6.3 +/- 0.1.

Examine the arteries periodically. Eliminate excessive kinking to allow for even flow distribution across the manifold. The arteries change in appearance within 20 minutes from a pinkish to a silvery color. The lumen dilates somewhat and the length of the

arteries increases about 35% during digestion. Do four full water rinses at the end of digestion.

For the stop bath, dissolve 20 grams of sodium chlorite into 20 liters of H_2O. Shut off the pumps and stirrer. Open drain valve and allow the tank to empty. Close the drain valve and fill the reactor with 20 liters of H_2O. Turn on the pumps and stirrer and allow to equilibrate for several minutes. Repeat for a total of four rinses.

Shut off the pumps and stirrer and open the drain valve to allow the tank to empty. Close the drain valve and fill the reactor with the stop bath solution. Start the pumps and stirrer. Run the stop bath for 15-20 minutes to inactivate any residual enzyme. Drain the reactor and perform four full rinses.

Dissolve 1680.2 grams of sodium bicarbonate in H_2O and dilute to 20 liters. Shut off the pumps and stirrer and drain the last rinse out of the tank. Fill the reactor with the 20 liters of the carbonate solution. Turn on the pumps and stirrer and allow to equilibrate for 15 minutes.

Weigh 142.8 grams of succinic anhydride. Add ½ of the anhydride to the reactor after the 15 minute equilibration time. Disperse the crystals into the solution so that the stirrer can mix them throughout the tank. Add the second half of the anhydride 30 minutes after the first addition. The charging should take another 60-90 minutes. Charging is complete when all of the crystals are dissolved and bubbles are no longer forming in the solution. When charging is done, stop the pumps and stirrer, drain the tank and perform four full rinses.

Measure 8.0 liters of H_2O and place in a clean carboy. Weigh out 112.0 g of sodium citrate and add to

the carboy. Weigh out 4.0 g of citric acid and add to the carboy. Place a magnetic stir bar in the carboy. Place the carboy on a stir plate and mix until all solids are dissolved. Citrate concentration should be 50 mM consisting of 95.2% sodium citrate and 4.8% citric acid.

Measure 1.6 ml of 50% glutaraldehyde and add to the citrate buffer while it is still on the stir plate. Allow solution to equilibrate for 10-15 minutes. Test pH and adjust as required to 6.40 +/- 0.05 using 6M NaOH or 6M HCl.

Place a clean fixation tank under the exhaust hood. Place a fixation rack into the fixation tank (2 levels for a standard batch). Transfer 0.01% glutaraldehyde solution from the carboy to the fixation tank. Maintain crosslinking conditions for up to approximately 120 hours, changing the glutaraldehyde solution periodically, i.e., every 24 hours.

Remove the manifold from the reactor. Using a clean scissors, cut an artery on the distal side of the collateral ulnar bifurcation. Place the artery in a beaker of demineralized water. Repeat above steps until all arteries have been removed from the manifold.

Remove one graft from the beaker. Extend the graft fully length-wise. Place the graft on a proper size mandrel and extend it fully length-wise. Place the mandrel with the graft into a spot on the fixation rack in the fixation tank. Repeat above steps until all grafts have been put on mandrels and placed in the fixation tank. Note time into the fixation tank and cover the tank.

When the grafts have in been the fixation solution for 24 hours, prepare a fresh batch of 0.01% glutaraldehyde and transfer the solution to a clean

fixation tank. Transfer the entire fixation rack (including grafts) from the existing fixation tank to the fresh glutaraldehyde solution. Discard the old glutaraldehyde. Place the tank with the fresh glutaraldehyde under the exhaust hood. Cover the fixation tank. Change the solution after each 24 hour interval.

Measure 7.68 liters of demineralized water and place in a clean carboy. Weigh out 112.0 g of sodium citrate and add to the carboy. Weigh out 4.0 g of citric acid and add to the carboy. Place the carboy on a stir plate and mix until all solids have been dissolved. Citrate concentration should be 50 mM, consisting of 95.2% sodium citrate and 4.8% citric acid.

Measure 320 ml of 50% glutaraldehyde and add to the citrate buffer while it is still on the stir plate. Allow the solution to equilibrate for 10-15 minutes. Test pH and adjust as required to 6.40 +/- 0.05 using 6M NaOH or 6M HCl. Transfer glutaraldehyde solution to a clean fixation tank prior to the sterilization process.

After approximately 120 hours of fixation, transfer the grafts to a 2% glutaraldehyde solution. Place fixation tank under the exhaust hood. Cover the fixation tank. Allow grafts to remain in the 2% glutaraldehyde for 4-5 hours.

Example 10.

Biological tissues can be produced as follows:

1. individual tissue valves are exposed to 0.01%, 0.02%, 0.03%, 0.04%, 0.05%, 0.06%, 0.07%, 0.08%, or 0.09% glutaraldehyde, buffered in either citrate (at pH 6.4) or HEPES (at pH 7.4), at 20°C for 2, 4, 5, 7, 9, or 10 days.

The crosslinked biological tissue can then be initially sterilized in a multi-component sterilant of 2% glutaraldehyde, 3% formaldehyde, and 20% ethanol in an aqueous buffer. The initially sterilized biological tissue may then be subjected to ethanol extraction (60% ethanol), optionally including 0.1M $AlCl_3$, and a 24 hour wash in saline.

The biological tissue is then packaged in a saline solution and subjected to terminal sterilization by exposing the tissue to e-beam radiation.

While the invention has been described in some detail by way of illustration and example, it should be understood that the invention is susceptible to various modifications and alternative forms, and is not restricted to the specific embodiments set forth. It should be understood that these specific embodiments are not intended to limit the invention but, on the contrary, the intention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the invention.

WHAT IS CLAIMED IS:

1. A method for treating a biological tissue comprising:

exposing a biological tissue to less than about 0.2% by volume crosslinking solution.

2. The method of claim 1 wherein the crosslinking solution is glutaraldehyde.

3. The method of claim 1 wherein the crosslinking solution is about 0.01% to about 0.099% by volume glutaraldehyde.

4. The method of claim 1 wherein the biological tissue is a collagenous material.

5. The method of claim 4 wherein the biological tissue is selected from the group consisting of bovine pericardium, porcine aortic valves, dura mater, human umbilical veins, vascular tissue derived from a variety of mammalian sources, and cardiac valve homografts.

6. The method of claim 1 further comprising exposing a crosslinked biological tissue to a bioburden reduction agent.

7. The method of claim 1 further comprising exposing the crosslinked biological tissue to an anticalcification reagent.

8. The method of claim 7 including exposing the crosslinked biological tissue to an anticalcification solution selected from the group consisting of at least one aluminum salt, a solution containing at least 50% ethanol, or combinations thereof.

9. The method of claim 1 further comprising sterilizing the crosslinked biological tissue.

10. The method of claim 6 further comprising sterilizing the crosslinked biological tissue.

11. The method of claim 8 further comprising sterilizing the crosslinked biological tissue.

12. The method of claim 9 wherein sterilizing the crosslinked biological tissue includes exposing the crosslinked biological tissue to e-beam particle bombardment.

13. A stabilized biological tissue comprising a biological tissue which has been exposed to less than about 0.2% by weight crosslinking solution, and having a shrink temperature from about 80°C to about 90°C.

14. A method for preparing a tissue for implantation comprising exposing a tissue to less than about 0.2% by volume crosslinking solution, and sterilizing the crosslinked tissue.

15. A method for packaging a tissue comprising exposing a tissue to less than about 0.2% by volume crosslinking solution, and exposing the tissue to electron beam radiation.

16. A biological tissue comprising a biological tissue which has been exposed to less than about 0.2% by volume crosslinking solution.

17. The biological tissue of claim 16 wherein the biological tissue has been exposed to about 0.01% to about 0.099% by volume glutaraldehyde.

18. A method for treating a biological tissue comprising exposing a biological tissue to from about 0.01% to about 0.099% by volume glutaraldehyde.

19. A method for treating a biological tissue comprising exposing a biological tissue to from about 0.01% to about 0.099% by volume glutaraldehyde, and exposing the biological tissue to an anticalcification solution selected from the group consisting of at least one aluminum salt, a solution containing at least 50% ethanol, or combinations thereof.

20. A biological tissue comprising a biological tissue which has been exposed to about 0.01% to about 0.099% by volume glutaraldehyde.

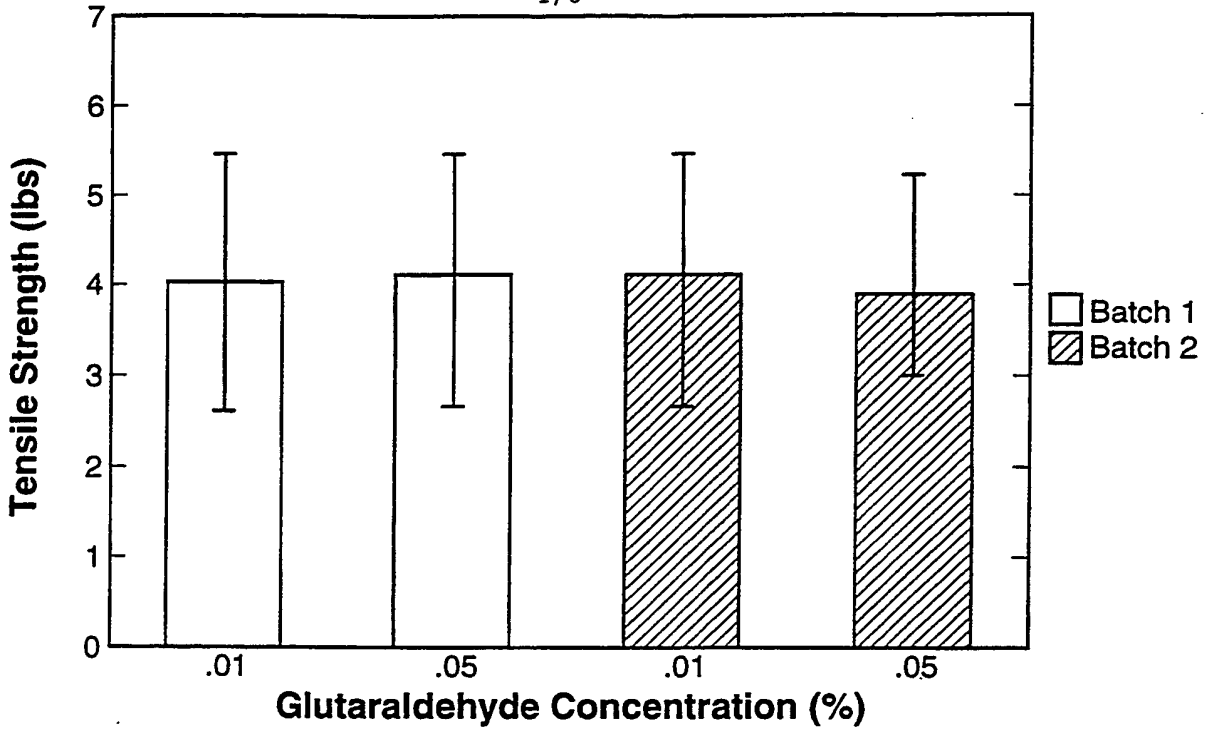

FIG. 1 Batch 1, 0.01%: n = 57 Batch 1, 0.05%: n = 48
Batch 2, 0.01%: n = 42 Batch 2, 0,05%: n = 36

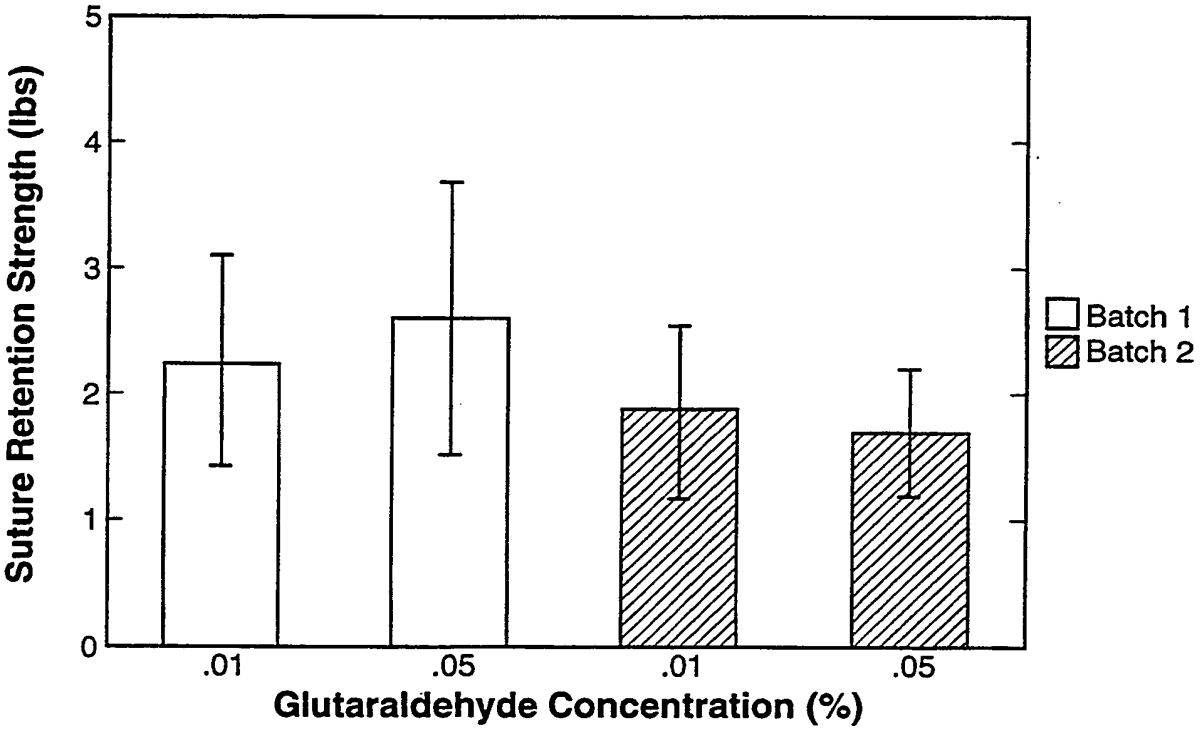

FIG. 2 Batch 1, 0.01%: n = 57 Batch 1, 0.05%: n = 48
Batch 2, 0.01%: n = 42 Batch 2, 0,05%: n = 36

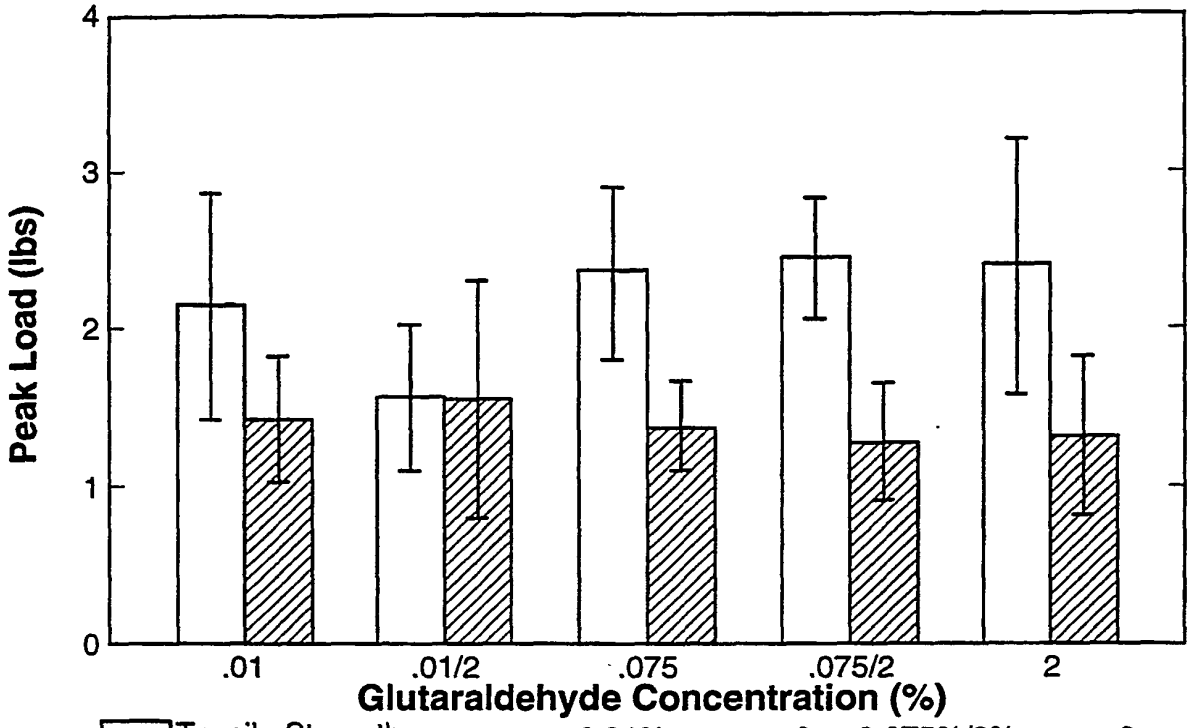

FIG. 3

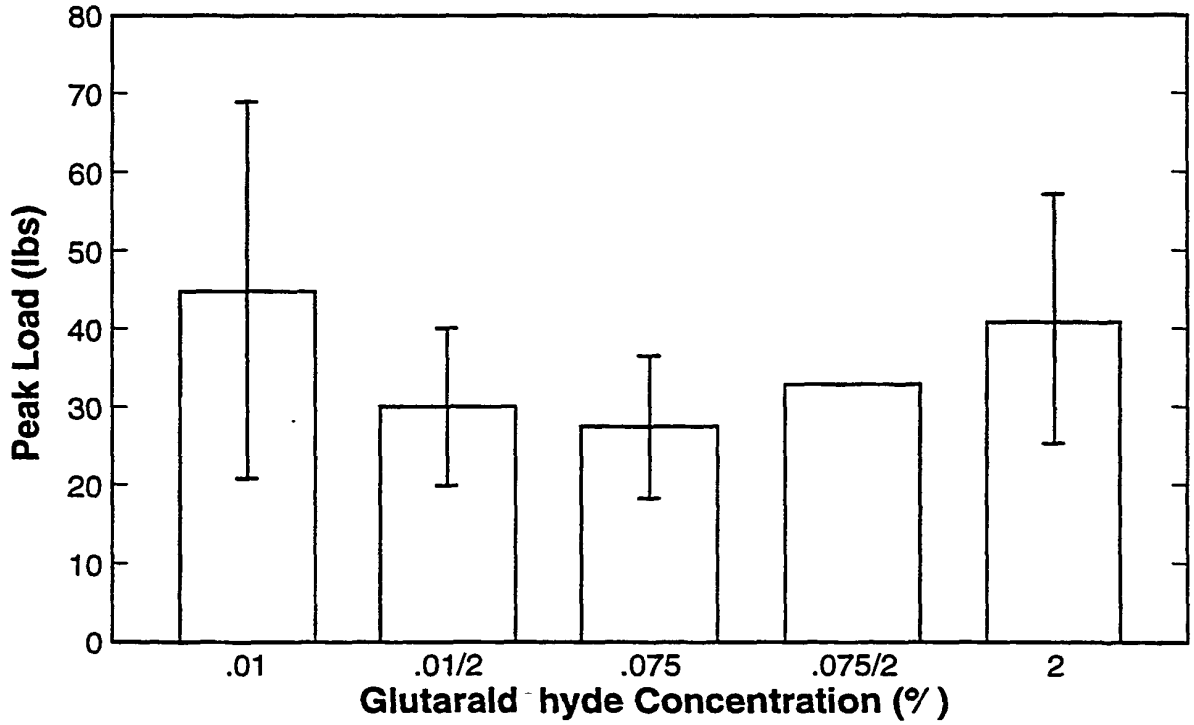

FIG. 4

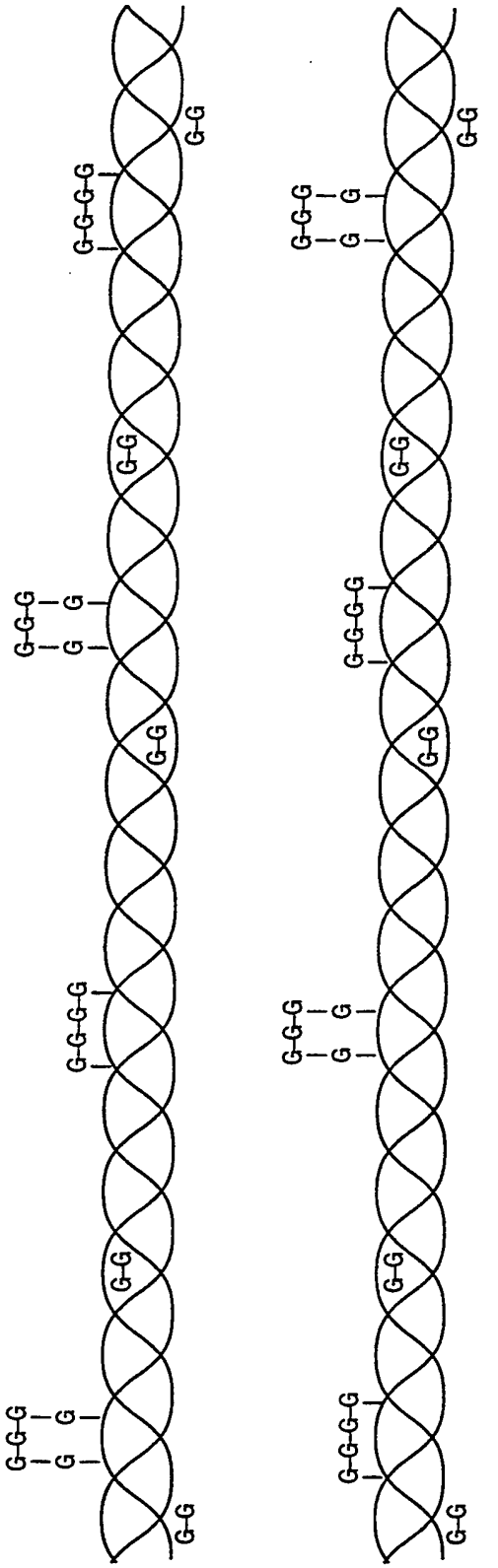

FIG. 5A

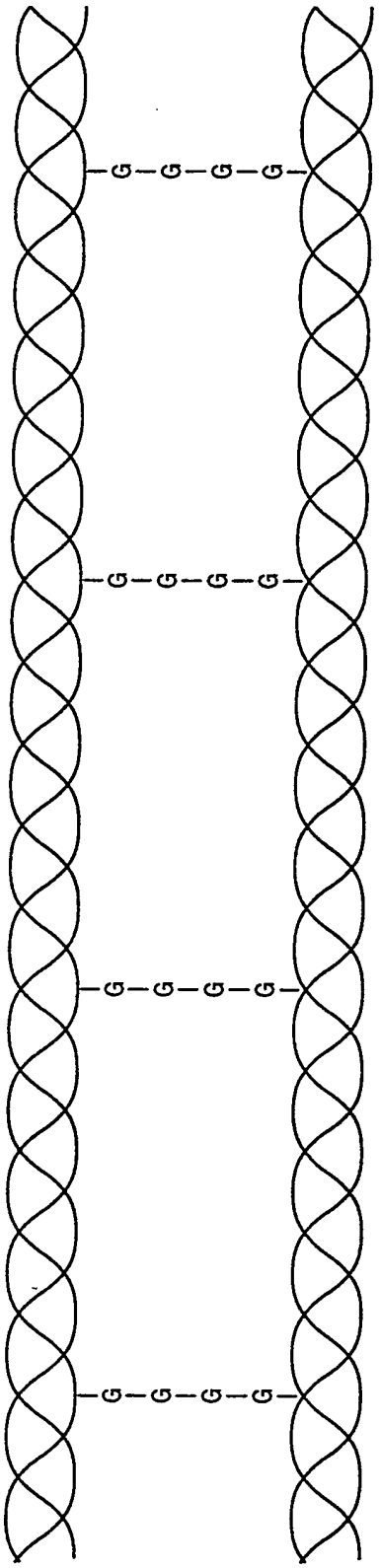

FIG. 5B

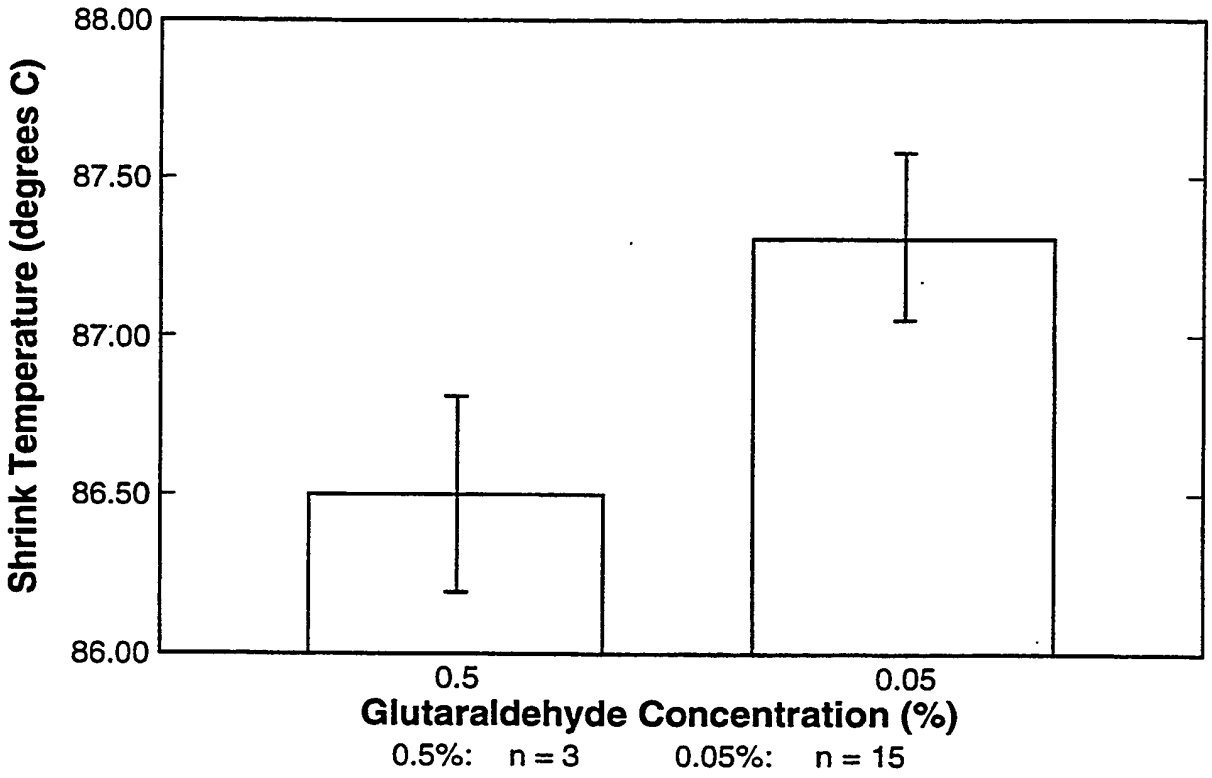

FIG. 6

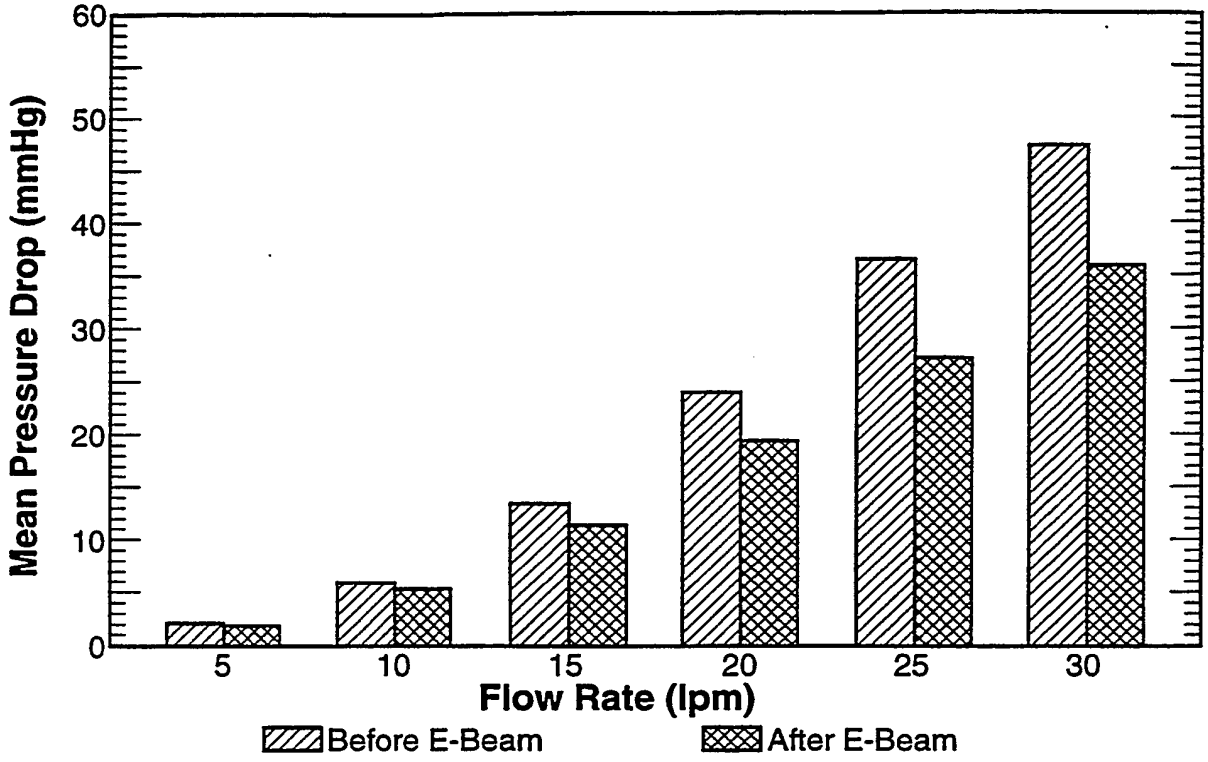

FIG. 7

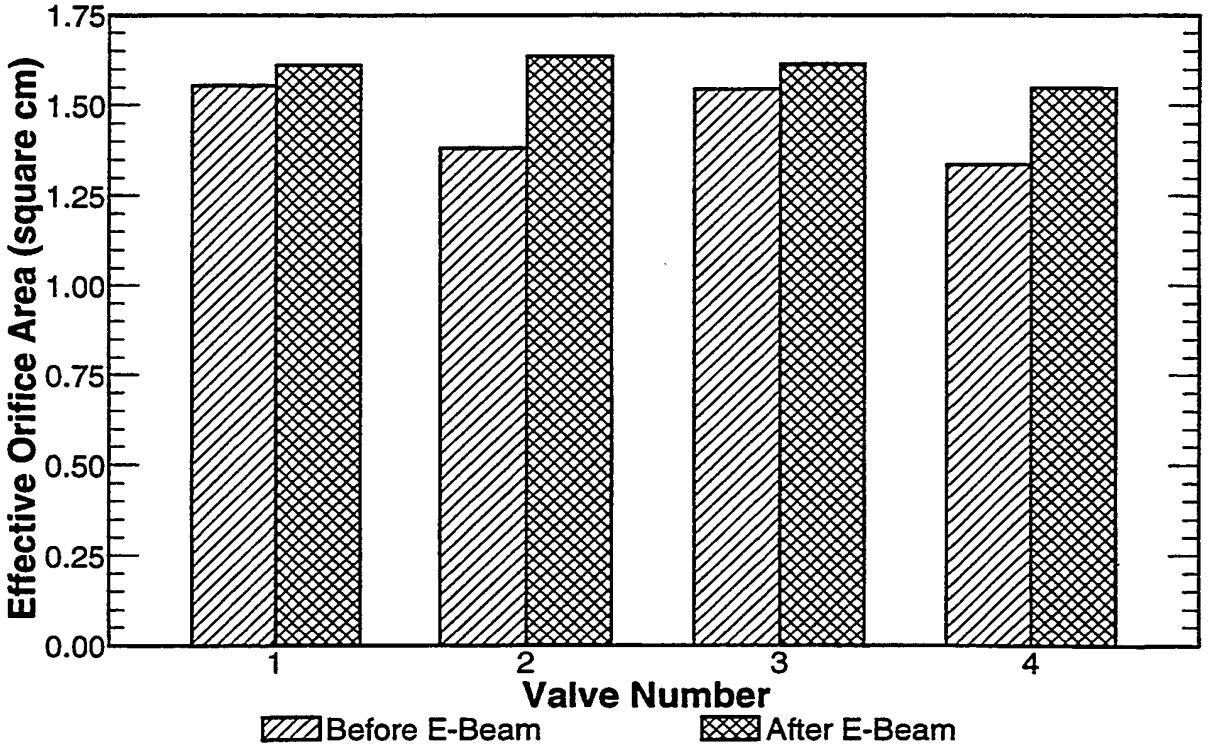

FIG. 8

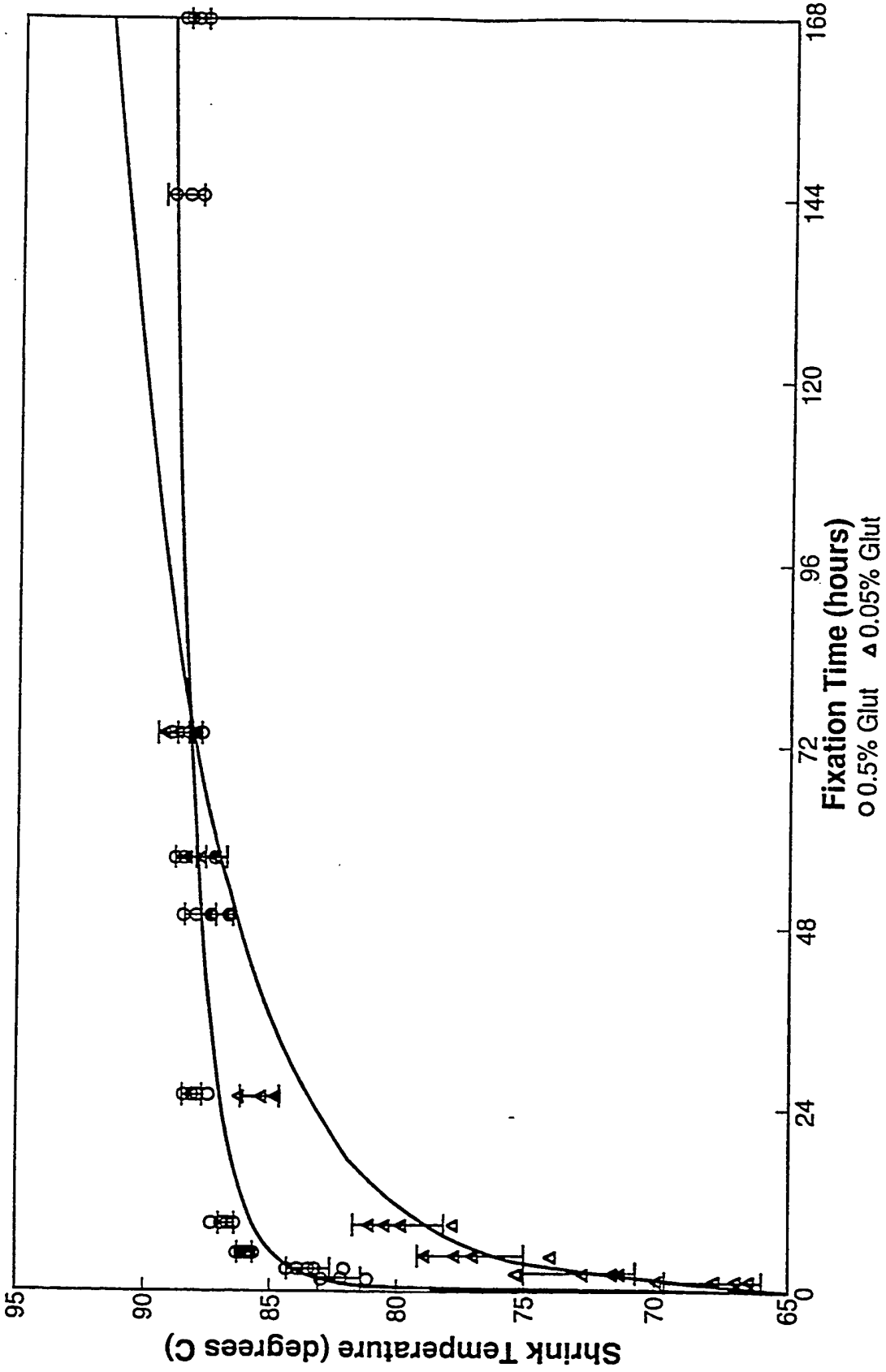

FIG. 9

INTERNATIONAL SEARCH REPORT

Inter[illegible]ional application No.
PCT[illegible] 95/07679

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61L 27/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

REGISTRY, CAPLUS, EPODOC, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP, A1, 0172716 (SHILEY INCORPORATED), 26 February 1986 (26.02.86), page 3, 2§, Claims 2-4,8	1-8,16-20
X	STN International, File CAPLUS, CAPLUS accession no. 1983:458864, Cooke, A. et al: "An in vitro cytotoxicity study of aldehyde-treated pig dermal ***collagen***", Br. J. Exp. Pathol. (1983), 64(2), 172-6	1-4,16-18,20
A	US, A, 4405327 (ELISABETH M. POLLOCK), 20 Sept 1983 (20.09.83), column 2, line 52 - line 58, abstract	1-11,14-20

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 Sept 1995	24. 10. 95
Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer SOFIA NIKOLOPOULOU

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US 95/07679

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0172716	26/02/86	AU-B- 554119	07/08/86
		AU-A- 4613185	27/03/86
		CA-A- 1254839	30/05/89
		JP-B- 1059241	15/12/89
		JP-C- 1587350	19/11/90
		JP-A- 61056101	20/03/86
US-A- 4405327	20/09/83	EP-A,A,A 0103946	28/03/84
		JP-A- 59037945	01/03/84